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Schlesinger, Martin ; Roblek, Marko ; Ortmann, Katrin ; Naggi, A ; Torri, G ; Borsig, L ; Bendas, Gerd

Abstract: Introduction: Heparin is known to efficiently attenuate metastasis in various tumour models by different mechanisms including inhibition of tumour cell contacts with soluble and cellular components such as inhibition of heparanase or P- and L-selectin. We recently showed that heparin efficiently binds to VLA-4 integrin in melanoma cells in vitro. Here we describe VLA-4 integrin as a mediator of melanoma metastasis that is inhibited by the low molecular weight heparin (LMWH) Tinzaparin. Materials and Methods: sh-RNA-mediated knock-down of VLA-4 integrin in B16F10 murine melanoma cells (B16F10-VLA-4kd) was performed and cell binding characteristics were investigated in vitro. Experimental metastasis of B16F10-VLA-4kd and B16F10 cells and interference by Tinzaparin were analysed in mice. Results: VLA-4 knock-down of B16F10 cells resulted in loss of VCAM-1 binding, but preserved the capacity to bind platelets through P-selectin. The observed reduced metastasis of B16F10-VLA-4kd cells confirmed the role of VLA-4 in this process. However, loss of melanoma VLA-4 function hardly further affected reduction of metastasis in P-selectin deficient mice. Tinzaparin treatment of mice injected with B16F10 and B16F10-VLA-4kd cells significantly reduced metastasis suggesting its potential to block both P- and L-selectin and VLA-4 in vivo. The use of N-acetylated heparin, which has no VLA-4 binding activity but blocks P- and L-selectin was less efficient than Tinzaparin in mice injected with B16F10 cells and B16F10-VLA-4kd cells. Conclusion: These findings provide evidence that heparin inhibits experimental melanoma metastasis primarily by blocking VLA-4 and P-selectin.

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The role of VLA-4 binding for experimental melanoma metastasis and its inhibition by heparin

Martin Schlesinger^{1,4,*}, **Marko Roblek**^{2,4}, **Katrin Ortmann**¹, **Annamaria Naggi**³,
Giangiaco Torri³, **Lubor Borsig**^{2,5}, **Gerd Bendas**^{1,5},

(1) Department of Pharmacy, Rheinische Friedrich Wilhelms University Bonn, 53121 Bonn, Germany

(2) Institute of Physiology & Zürich Center for Integrative Human Physiology, University of Zurich, CH-8057 Zurich, Switzerland

(3) “G. Ronzoni” Institute for Chemical and Biochemical Research, 20133 Milano, Italy

(4) contributed equally to this work

(5) authors share senior authorship

* Correspondence to: Dr. Martin Schlesinger

Department of Pharmacy
Rheinische Friedrich Wilhelms University Bonn
An der Immenburg 4
53121 Bonn
Germany
Tel.: +49 228 735238,
Fax: +49 228 734692
E-mail: mschlesi@gmx.de

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Running title: Heparin inhibits VLA-4 mediated metastasis

SUMMARY

Introduction: Heparin is known to efficiently attenuate metastasis in various tumour models by different mechanisms including inhibition of tumour cell contacts with soluble and cellular components such as inhibition of heparanase or P- and L-selectin. We recently showed that heparin efficiently binds to VLA-4 integrin in melanoma cells *in vitro*. Here we describe VLA-4 integrin as a mediator of melanoma metastasis that is inhibited by the low molecular weight heparin (LMWH) Tinzaparin.

Materials and Methods: sh-RNA-mediated knock-down of VLA-4 integrin in B16F10 murine melanoma cells (B16F10-VLA-4kd) was performed and cell binding characteristics were investigated *in vitro*. Experimental metastasis of B16F10-VLA-4kd and B16F10 cells and interference by Tinzaparin were analysed in mice.

Results: VLA-4 knock-down of B16F10 cells resulted in loss of VCAM-1 binding, but preserved the capacity to bind platelets through P-selectin. The observed reduced metastasis of B16F10-VLA-4kd cells confirmed the role of VLA-4 in this process. However, loss of melanoma VLA-4 function hardly further affected reduction of metastasis in P-selectin deficient mice. Tinzaparin treatment of mice injected with B16F10 and B16F10-VLA-4kd cells significantly reduced metastasis suggesting its potential to block both P- and L-selectin and VLA-4 *in vivo*. The use of *N*-acetylated heparin, which has no VLA-4 binding activity but blocks P- and L-selectin was less efficient than Tinzaparin in mice injected with B16F10 cells and B16F10-VLA-4kd cells.

Conclusion: These findings provide evidence that heparin inhibits experimental melanoma metastasis primarily by blocking VLA-4 and P-selectin.

Keywords: cell adhesion, heparin, melanoma cell metastasis, selectins, VLA-4

Abbreviations:

FCS, fetal calf serum; FAK, focal adhesion kinase; LMWH, low molecular weight heparin; mAb, monoclonal Antibody; PBS, phosphate-buffered saline; PE labeled, Phycoerythrin labeled; PKC α , protein kinase C alpha; UFH, unfractionated heparin; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4

INTRODUCTION

The metastatic spread of solid tumours is the major cause for the poor outcome of cancer patients. The settlement of tumour cells in distant organs is commonly described as a metastatic cascade. Tumour cells detach from the primary tumour, migrate through the extracellular matrix, invade the blood circulation, interact with blood constituents, adhere to the endothelium, extravasate, and proliferate in distant tissues. Adhesion molecules mediate the critical interaction between tumour cells and the vascular endothelium as well as cellular and soluble blood components.

The selectin family of vascular adhesion receptors has early been identified as key players in the metastatic process [1–3]. In the early phase of hematogenous metastasis P-selectin expressed on activated platelets and endothelium contributes to platelet-rich tumour cell emboli formation resulting in protection of tumour cells from innate immune system [4,5]. L-selectin expressed by leukocytes facilitates the interaction of the tumour microemboli with the endothelium [6,7].

Integrins are a larger family of adhesion receptors that facilitate the interaction of tumour cells with vasculature and circulating cells in the metastatic course. However, the exact role of integrins in this process remains under investigation. One member of this family is the very late activation antigen-4 (VLA-4, $\alpha_4\beta_1$) expressed under physiological conditions on different subtypes of leukocytes, but is also found on melanoma, osteosarcoma, and rhabdomyosarcoma cells [8–11]. VLA-4 is able to bind to its ligand vascular cell adhesion molecule-1 (VCAM-1) expressed by activated endothelium thereby mediating adhesion and a subsequent transmigration of tumour cells [8,12]. Accordingly, VLA-4 contribution to metastasis has been demonstrated in several studies [13–15]. In addition, the enhanced expression of VLA-4 on melanomas correlates with poor clinical outcome [16–18]. A lot of efforts have been invested into inhibition of adhesion receptors of the selectin or integrin family and thereby intervention in metastasis [19].

Heparin is routinely administered to cancer patients for an antithrombotic prophylaxis due to their increased risk for cancer-associated thrombosis. In a series of prospective clinical trials heparin has been shown to prolong survival of cancer patients especially those with initial better prognosis [20–24]. These effects were shown not only to be dependent on the antithrombotic properties of heparin, but on the capacity to reduce metastasis by inhibition of P- and L-selectin [25, 26]. In addition, several other anti-metastatic activities of heparin have been described including (i) inhibition of angiogenesis via stimulation of tissue factor pathway inhibitor (TFPI) release from the endothelium [27], (ii) modification of growth factors and chemokines binding to their receptors [28], (iii) inhibition of tumour cell secreted heparanase [29], (iv) potential interference with chemoresistance of cancer cells [30].

Furthermore, LMWH is apparently affecting intracellular signaling to downregulate the expression of FAK/p53 and PKC α which gave rise to a decrease in cell-substratum interface area, an alleviated melanoma cell migration, and an altered cell morphology [31,32].

Earlier we demonstrated that heparin also binds to the integrin VLA-4 on melanoma cells and impedes the interaction with its endothelial ligand VCAM-1 *in vitro*. We performed several structure-activity correlations for several non-anticoagulant heparin derivatives based on chain length and sulfation degree [33]. Particularly, a *N*-acetylated heparin derivative was completely incapable to inhibit the VLA-4 adhesion but binds P- and L-selectin [33]. However, a LMWH such as Tinzaparin, appears most suitable to cover the anti-adhesive activities that is in line with the other anti-metastatic activities including anticoagulative effects.

Although the role of VLA-4 in melanoma metastasis has been approached before [12–15], the potential of heparin to inhibit metastasis through blocking VLA-4 interactions has not been explored until now. In the present study we demonstrate for the first time that heparin interference with VLA-4 reduces the metastatic dissemination of melanoma cells. Inhibition with LMWH Tinzaparin or a non-VLA-4 binding heparin derivative provides evidence that the VLA-4 inhibition is critical for attenuation of experimental melanoma metastasis.

MATERIALS and METHODS

Chemicals and proteins

Recombinant human VCAM-1-Fc chimera, anti-human P-selectin mAb, control mAb, anti-mouse integrin CD49d mAb (Phycoerythrin (PE)-labeled), and thrombin receptor activator peptide 6 (TRAP-6) were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Tinzaparin was obtained from LEO Pharma GmbH (Neu-Isenburg, Germany). Bovine serum albumin (BSA), sepharose[®] 4B, and cyanuric chloride were from Sigma-Aldrich (Deisenhofen, Germany), and Calcein-AM from Molecular Probes Europe BV (Leiden, Netherlands). The *N*-acetylated heparin derivatives were prepared as previously described [34].

Cell culture

The murine melanoma cell line B16F10 was kept in D-MEM with 4.5 g/L glucose supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine and 1% (v/v) penicillin-streptomycin solution. To reduce the constitutive VLA-4 expression, a sh-RNA technique was applied for a continuous downregulation. B16F10 cells were transfected with a commercial available shRNA-plasmid (Mission[®] shRNA, Sigma-Aldrich), which was amplified in *E. coli* and isolated by Maxi-Prep (Sigma-Aldrich). Additionally, cells were transfected with a control plasmid merely coding for puromycin resistance. Lipid-based transfection was performed with FuGene[®]6 (Roche Applied Science), DNA-lipid ratio has previously been determined by GFP-plasmid transfection. To select transfected cells expressing anti-VLA-4-shRNA, a permanent puromycin treatment was performed. The VLA-4 expression level in transfected cells was ascertained by flow cytometry (FACS Calibur, BD Heidelberg, Germany) using a PE-labeled anti-mouse integrin CD49d mAb.

bEnd.3 cells were cultivated in D-MEM with 4.5 g/L glucose supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine and 1% (v/v) penicillin-streptomycin solution.

Platelet isolation and cell platelet interaction

Blood was collected from healthy volunteers in 15 mL tubes containing 1.6 mg potassium EDTA/mL blood. Afterwards, blood was centrifuged at 200 g for 20 min at 20°C to gain platelet-rich plasma. Separation of platelets from plasma proteins was conducted by gel filtration through a sepharose[®] 4B column. For adhesion experiments and for viability determination, platelets were labeled with 5 µg/5 mL Calcein-AM for 30 min at 37°C. Platelets were activated using TRAP-6 at a concentration of 5 µM for 15 min at 37°C. To compare the adhesion of B16F10 wild type (B16F10) and B16F10 VLA-4 knock-down (B16F10-VLA-4kd) cells to platelets either under static or dynamic conditions the following protocols were applied: i) Platelets were labeled, activated as described above, adjusted to a concentration of 1×10^8 platelets, and added to 1×10^6 /100 µL cell suspension. After addition

of 5 µg of anti-P-selectin mAb, control mAb, or 500 µg Tinzaparin, the mixture was incubated for 5 min at 37°C under gently shaking. After dilution with 800 µL PBS, the cell platelet interaction was determined by flow cytometry.

ii) Labeled and activated platelets (1×10^8) were added to a confluent layer of B16F10 or B16F10-VLA-4kd cells in 24-well cell culture plates alone or in presence of mAbs or Tinzaparin, mild shaken for 15 min, washed twice with PBS and the adherent platelets were counted with fluorescence microscopy.

Adhesion under static conditions

Calcein-labeled B16F10, B16F10 control cells, or B16F10-VLA-4kd cells (1×10^5) cells were added to a confluent layer of bEnd.3 cells in a total volume of 200 µL PBS in a 96 well plate and allowed to sediment for 15 min under gently shaking conditions. After washing twice with 200 µL PBS, the adherent cells were quantified using a POLARstar Galaxy plate reader (BMG Labtech).

Transmigration of B16F10

Migration assays were performed in a 96 well transwell containing 8 µm pore-size inserts (Corning, Corning, NY) coated with gelatine (Sigma-Aldrich, Deisenhofen, Germany), overgrown with bEnd.3 endothelial cells. bEnd.3 cells (1×10^4) were cultivated for 2 days to form a confluent layer in 50 µL medium. 5×10^4 B16F10, B16F10 control, or B16F10-VLA-4kd cells in 50 µL medium containing 1% (v/v) FCS were cultivated in the top compartment and exposed to chemotactic attraction by 10% (v/v) FCS in the medium in the bottom chamber.

The chambers were incubated at 37°C in humidified air with 5% CO₂ for 24 hours. Migrated cells were labeled with 2 mM Calcein-AM (Molecular Probes) in 0.02 % EDTA solution (Sigma-Aldrich, Deisenhofen, Germany) at 37°C for 1 hour and quantified in a POLARstar Galaxy plate reader (BMG Labtech). Results are expressed as mean \pm SD from six technical replicates. Cells were incubated with neutralizing anti-VLA-4 antibodies (R&D Systems) for 5 minutes prior start of the experiment.

Flow chamber assay

The interaction of B16F10 and B16F10-VLA-4kd cells with the ligand VCAM-1 was investigated under physiological flow conditions in a parallel plate flow chamber as described before [35,36]. Briefly, VCAM-1 Fc chimera (0.2 µg) was coated on glass slides which were incorporated into a parallel plate flow chamber and mounted on an inverted microscope. Flow medium, PBS (pH 7.4), was applied with a shear rate of about 200 s⁻¹.

B16F10 and B16F10-VLA-4kd cells (1×10^6) were suspended in FCS-free medium and stimulated with 1 mM Mn^{2+} for 5 min at 37°C (stimulated cells) in comparison to unstimulated cells. Activation of VLA-4 with Mn^{2+} is an experimentally accepted approach to induce strong upregulation of integrin binding affinity as alternative to activation by mediators like chemokines, *in vitro* [37].

After application into the flow chamber, cell adhesion was observed for a period of 5 s while video sequences were recorded with the camera CSC-795 (Pacific Corporation, Tokyo, Japan). Videos were evaluated with Imagoquant MultiTrack-AVI-2 software (Mediquant GmbH, Lützen, Germany). In some experiments Tinzaparin (50 µg) was added to test its inhibitory capacity towards the VLA-4/VCAM-1 interaction.

Animal experiments

P-selectin-deficient mice (P-sel^{-/-}) in a C57BL/6J background and control wild-type (wt) C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6J or P-sel^{-/-} mice were intravenously injected with 67 IU of Tinzaparin or 150 µg of *N*-acetylated heparin, followed by injection of B16F10 or B16F10-VLA-4kd cells (1.5×10^5) through the tail vein 10 min later. After 14 days mice were euthanized, metastatic foci were counted and macroscopic pictures of lungs were taken. Mice were fed standard chow and water ad libitum and maintained on a 12-hour light/dark cycle. All animal protocols were approved by the Zürich Kantonal committee.

Statistics

Data are represented as mean \pm standard deviation of at least three independent experiments. Comparisons were performed by the unpaired Student's t-test.

RESULTS

Impact of VLA-4 on B16F10 cell adhesion in vitro and transmigration

To investigate the relevance of VLA-4 for metastatic dissemination of tumour cells we chose the highly metastatic murine melanoma cell line B16F10 that constitutively expresses VLA-4. B16F10 cells strongly adhered to immobilized VCAM-1 under physiological flow conditions [35]. Using shRNA approach we prepared VLA-4 knock-down cells, B16F10-VLA-4kd, which displayed a VLA-4 downregulation of up to 40 % compared to the wild type cells as determined by flow cytometry (Fig. 1A). B16F10 cells transfected with a control plasmid (B16F10 control cells) as expected had no effect.

The functional consequence of reduced VLA-4 expression for binding to immobilized VCAM-1 was evaluated under dynamic flow conditions using a flow chamber, detecting the Mn^{2+} activated cells.

B16F10-VLA-4kd cells showed a significantly reduced binding compared to B16F10 cells (Fig. 1B). After 4 sec of flow, 80 % of the Mn^{2+} activated B16F10 cells still adhered to the VCAM-1 layer whereas only 20 % of the B16F10-VLA-4kd clone cells were still attached, which was similar to adhesion of unstimulated B16F10 cells (15 %). Accordingly, Mn^{2+} stimulated B16F10 control cells possessed a similar binding behaviour like B16F10 cells (79 %). Despite the incomplete down-regulation of VLA-4 expression, B16F10-VLA-4kd cells were unable to adhere to VCAM-1 under flow condition. This indicated that a minimum of VLA-4 density on the cell surface must be sustained to keep a complete adhesion function. Furthermore it became evident that unstimulated B16F10 cells retain a certain residual VLA-4 activity, since unstimulated B16F10-VLA-4-kd cells revealed a further reduction in binding to VCAM-1 after 4 s of flow (5 %) compared to unstimulated B16F10 cells.

Application of Tinzaparin revealed a complete block of VLA-4 adhesion receptors both on stimulated B16F10 and B16F10-VLA-4kd cells reducing the cell adhesion to unstimulated B16F10-VLA-4kd cells. To approach more physiological binding conditions, we further investigated the impact of VLA-4 downregulation on endothelial contact formation and transmigration of B16F10 melanoma cells.

In a first step the binding of B16F10 cells to a bEnd.3 endothelial layer was analysed under static conditions (Fig. 1C). Both, VLA-4 and P-selectin contribute to endothelial interaction of the B16F10 cells, which was confirmed by application of mAbs against VLA-4 and P-selectin. Accordingly, B16F10-VLA-4kd cells failed to adhere to the bEnd.3 layer and displayed binding comparable to the non-activated cells (0.57 ± 0.02 compared to 0.65 ± 0.15). Antibodies against VLA-4 and P-selectin reduced binding to bEnd.3 cells of both B16F10 and B16F10-VLA-4kd cells in a similar ratio, whereas P-selectin seems to contribute to cell adhesion to a greater extent than VLA-4. Tinzaparin, as an inhibitor of both VLA-4 and P-

selectin, blocked efficiently binding of B16F10 cells (0.35 ± 0.07). The expected predominance of Tinzaparin to block B16F10 binding compared with the antibodies was not displayed in this assay, apparently due to preincubation of B16F10 cells with Tinzaparin. Although VLA-4 on B16F10 cells can efficiently be blocked by Tinzaparin, it has to compete with high affinity P-selectin ligands on B16F10 cell for P-selectin binding on endothelial cells. Therefore a complete P-selectin inhibition is not ensured, as clearly evident in case of the B16F10-VLA-4-kd cells.

To simulate further steps in the metastatic process, we tested the transmigration efficiency of B16-VLA-4kd through endothelial cells (Fig. 1D). The B16F10-VLA-4-kd displayed a reduced transmigration rate (0.67 ± 0.02) compared with B16F10 cells (1.0 ± 0.1), which indicates the importance of VLA-4 for both, contact formation and transmigration.

Next we asked whether the VLA-4 downregulation has any impact on the selectin binding of B16F10-VLA-4kd clone cells. P-selectin and L-selectin were previously shown to facilitate metastasis of melanomas [29]. Therefore, we analysed the interaction of activated, P-selectin expressing platelets with B16F10 and B16F10-VLA-4kd clone cells (Fig. 2). First we analysed the platelet-melanoma interactions under static conditions using fluorescence microscopy (Fig. 2A). Platelet activation with TRAP-6 enhanced their binding to the cell layer of B16F10, B16F10 control cells and B16F10-VLA-4kd clone cells to a similar extent of approximately 60 platelets per view field. Administration of an anti-P-selectin mAb almost abrogated the platelet interaction with melanomas compared to a control mAb, thereby confirming the P-selectin dependency of platelet-melanoma cell interactions. Similarly, Tinzaparin, a known inhibitor of P-selectin, blocked the interactions of platelets with melanomas to the same extent as anti-P-selectin mAb. To ascertain that platelet interaction with tumour cells is also valid under dynamic conditions, flow cytometry experiments were performed. The data confirmed the results received under static conditions (Fig. 2B). B16F10, B16F10-VLA-4kd, and B16F10 control cells showed an identical binding to activated platelets, and the use of Tinzaparin or the P-selectin mAb (data not shown) reduced binding to melanomas.

Taken together, the tumour cell-platelet interaction is P-selectin mediated, sensitive to Tinzaparin and not affected by VLA-4 downregulation. Thus, we confirmed that the B16F10-VLA-4kd cells are suitable to differentiate between VLA-4-dependent and P-selectin-dependent binding which will enable us to study the inhibitory potential of heparin *in vivo*.

VLA-4 mediates experimental metastasis of B16F10

To evaluate the involvement of VLA-4 on B16F10 during hematogenous metastasis, we intravenously injected C57BL/J6 mice with 1.5×10^5 B16F10, B16F10-VLA-4kd cells, or

B16F10 control cells, respectively. Mice were terminated after 14 days and the number of pulmonary metastatic nodules was determined (Fig. 3). While mice injected with B16F10 or B16F10 control cells showed almost displaced lungs with metastasis, injection of B16F10-VLA-4kd cells resulted in a significant reduction of metastatic foci. These results clearly confirm VLA-4 as a key player in experimental B16F10 melanoma metastasis.

Differentiation between P-selectin and VLA-4-mediated melanoma metastasis

There is a large body of evidence that P-selectin and L-selectin facilitate metastasis of tumour cells when they are still in the circulation [4,6]. Therefore, we aimed to differentiate between the contribution of tumour cell-derived VLA-4 and selectin ligands to metastasis of B16F10 melanoma. We injected B16F10 or B16F10-VLA-4kd cells to P-selectin deficient mice. We observed significant attenuation of metastasis with both cells indicating that in the absence of P-selectin, VLA-4 presence or absence did not have a statistically significant effect on the reduced number of metastatic nodules (Fig. 4).

Tinzaparin as an inhibitor of P-selectin and VLA-4-mediated interactions

Next we assessed the anti-metastatic activity of heparin with the aim to compare VLA-4 inhibition to P-selectin inhibition. We recently provided evidence that LMWH Tinzaparin has a high affinity binding to P-selectin and VLA-4 in the low micromolar range [33].

We injected wild type mice with 67 IU of Tinzaparin 10 min prior to injection of B16F10 or B16F10-VLA-4kd cells. Tinzaparin treatment of mice almost abolished experimental metastasis of B16F10 and B16F10-VLA-4kd cells (Fig. 5). Next we injected P-selectin deficient mice with Tinzaparin prior to application of B16F10 or B16F10-VLA-4kd cells (Fig 4). Tinzaparin treatment further reduced metastasis to background levels indicating that other biological activities beyond adhesion (e.g. coagulation) are also part of antimetastatic treatment.

To further dissect the role of VLA-4 and P-selectin inhibition during metastatic seeding, we tested a *N*-acetylated heparin derivative, which failed to bind to VLA-4, but preserves P- and L-selectin binding capacity [29,33]. *N*-acetylated heparin treatment of mice injected with B16F10 cells was less efficient than Tinzaparin which emphasizes a metastasis-contributing role of melanoma derived VLA-4, as indicated in Fig.3. In addition, *N*-acetylated heparin treatment of mice injected with B16F10-VLA-4kd cells resulted in a further but not significant reduction of metastatic burden, however not to the level reached by Tinzaparin treatment. Together, these findings provided evidence that inhibition of P-selectin is the major anti-

metastatic activity of Tinzaparin, while inhibition of VLA-4-mediated interactions further blocks metastatic spread.

DISCUSSION

The anti-metastatic activities of heparin have been a matter of intensive research during the last decade and research on therapeutic potential is still ongoing. Several molecular mechanisms of heparin have been described and also have been experimentally confirmed. Inhibition of coagulation seems to be a basis for heparin activities, also in blocking metastasis. Despite a great variation in the tested tumour models, the blockade of P- and L-selectin appears also as an important target of heparin [25]. This opinion further underlined the importance of cellular adhesion receptors in the metastatic cascade and their role as therapeutic targets.

Compared to selectins, the receptor family of integrins is ubiquitously expressed by virtually all cells, and different integrin pairs are involved in variety of cellular processes [38]. However their role during cancer metastasis is less defined and studied.

Here we studied the role of VLA-4 integrin in melanoma metastasis and evaluated the inhibitory potential of heparin. Several melanoma cells express high levels of the integrin VLA-4 on their surfaces [11]. The relevance of VLA-4 interaction with VCAM-1 on the endothelium during metastatic spread has been shown by several groups in the early 90s [13–15]. Accordingly, VLA-4 was proposed as a target for interference in the metastatic cascade. Nevertheless, VLA-4 has attracted more attention as a target for autoimmune diseases like multiple sclerosis, where a blocking antibody has been approved for therapy [39].

Recently we provided evidence for a heparin capacity to bind VLA-4 [35]. Although we described numerous structure-activity relationships of heparin-VLA-4 binding [36] the relevance of this interaction has never been addressed *in vivo*. Here we show that VLA-4 on murine melanoma cell line B16F10 contributes dominantly to its experimental metastasis. Furthermore, we provide evidence for the first time that a LMWH - Tinzaparin - blocks VLA-4 *in vivo* thereby reducing the metastatic dissemination of melanoma cells. Although these findings have been conducted in an experimental metastasis assay, certain effects of metastasis formation (an impact of microenvironment) are not adequately reflected, these data allude to a novel physiologically relevant mechanism how heparin interferes with experimental metastasis. However, we acknowledge that a spontaneous metastasis model would be more relevant to the clinical setting.

We show that integrin and P-selectin are two adhesion mechanisms involved in metastatic spread. Our results indicate that in the P-sel^{-/-} background, VLA-4 only has a minimal and not significant additional contribution to the establishment of metastatic foci and that P-selectin is dominant over the integrin VLA-4 which was also confirmed under *in vitro* conditions.

VLA-4 mediates endothelial arrest of melanoma cells that is supported by P-selectin-mediated tumour cell emboli formation. Interestingly, Tinzaparin almost completely

attenuates metastasis of both wt B16F10 and B16F10-VLA-4kd cells. This is in agreement with the capacity of Tinzaparin to affect both P-selectin and VLA-4 binding. However, *N*-acetylated heparin has only capacity to bind P-selectin but not VLA-4 and is non-anticoagulant [33]. Accordingly, *N*-acetylated heparin attenuated metastasis of B16F10 cells less efficiently compared to Tinzaparin. Nevertheless a significant decrease in the number of metastatic foci was observed in mice injected with B16F10-VLA-4kd cells and treated with *N*-acetylated heparin blocking P-selectin compared to non-treated controls. These results indicate that both P-selectin and VLA-4 mediated interactions significantly contribute to metastatic spread of melanoma cells.

Tinzaparin treatment almost completely inhibited metastasis, indicating additional biological activity beyond inhibition of cell adhesion. Tumor-induced coagulation has a significant role in thrombus formation that is associated with cancer progression [40,41]. However, Tinzaparin treatment of mice at clinically relevant concentration was clearly beneficial over a specific anti-thrombotic treatment, Fondaparinux, strongly suggesting that inhibition of coagulation is not significantly contributing to anti-metastatic activity in a mouse model of experimental metastasis [42]. Here we provide a direct comparison of Tinzaparin with modified heparins (*N*-acetylated), which has reduced biological activities. Clearly, Tinzaparin has additional beneficial effect over *N*-acetylated heparin on metastasis, which will be the focus of further investigations.

Our findings describe an important role of integrin VLA-4 engagement on tumour cells by blood components and endothelium during hematogenous spread. However, VLA-4 seems to be also involved in melanoma transmigration through the endothelial cells *in vitro*. Since integrins are bidirectional signalling molecules, heparin-integrin interaction may also have further consequences beyond the adhesion. Integrins are also involved in the crosstalk with numerous classes of growth factor receptors and cytokine receptors and are able to mediate proangiogenic and proliferative signals to the tumour cells [43–47]. On contrary, integrins can also initiate pro-survival and pro-tumourigenic as well as pro-apoptotic signals [44,48,49]. The activation of each pathway depends on the ligation status of the integrins by a given cell. In cells in which most of the integrins are ligated, pro-survival signals were reported whereas pro-apoptotic signals were found in unligated cells [50,51]. The role of heparin in this complex network will be addressed in future studies.

Concluding, our data introduce VLA-4 as a key player in B16F10 melanoma experimental metastasis, which can efficiently be blocked by the LMWH Tinzaparin, whereby reducing the metastatic burden. Furthermore, not only VLA-4 expressing tumour cells should be addressed by these findings. Recent reports revealed further functionalities of VLA-4 and VCAM-1 in the metastatic microenvironment, which are beyond a direct tumour cell contact with the endothelium. For instance, in VCAM-1 expressing breast tumour cells a pro-survival

signalling is induced upon binding to macrophages via VLA-4. In addition, VCAM-1 positive breast tumour cells metastasizing to the bones decoy VLA-4 osteoclast progenitors whereby promoting destruction of the bone matrix [52,53] Also the recruitment of myeloid cells that promote pulmonary metastasis in mice has revealed to be VLA-4/VCAM-1 dependent [54]. Hence, VLA-4 is involved in the metastatic niche in multiple facets, so our findings give a novel input for therapeutic strategies to target VLA-4 dependent metastatic pathways by heparin.

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Legends to Figures:

Figure 1: Functional consequences of a VLA-4 knock-down on adhesion and transmigration of B16F10 cells to immobilized VCAM-1 under flow conditions. **A)** B16F10, B16F10 control cells, and B16F10-VLA-4kd were incubated with an anti-mouse VLA-4 mAb (PE labeled) and investigated with flow cytometry. B16F10 and control cells expressed comparable levels of VLA-4 on their surfaces, whereas B16F10-VLA-4kd display a VLA-4 downregulation by 40% compared to B16F10 cells. **B)** B16F10, B16F10 control cells, or B16F10-VLA-4kd cells ($1 \times 10^6/100 \mu\text{L}$ medium) were put into the flow chamber (in some cases after stimulation with 1 mM Mn^{2+}) and allowed to sediment for 5 min. After this period, the flow was started and the adhesion behaviour was monitored over the indicated time range. B16F10 and B16F10 control cells displayed a similar adhesion after Mn^{2+} stimulation, whereas B16F10-VLA-4kd cells showed a significantly reduced binding approximating the level of unstimulated B16F10 cells. Application of Tinzaparin also reduced the binding to the same level of unstimulated B16F10-LAV-4kd cells. **C)** Calcein-labeled B16F10 or B16F10-VLA-4kd cells (1×10^5) cells were added to a confluent layer of bEnd.3 cells in a total volume of $200 \mu\text{L}$ PBS in 96 well plate and allowed to sediment for 15 min. After washing twice with PBS, the adherent cells were quantified in a plate reader. B16F10-VLA-4kd cells as well as the application of an anti-VLA-4 mAb, anti-P-sel-mAb, or Tinzaparin displayed a reduced binding to bEnd.3 cells. **D)** Transmigration of B16F10 or B16F10-VLA-4kd cells through a confluent layer of bEnd.3 cells was determined using a boyden chamber approach with a FCS gradient (for details see Materials and Methods). A VLA-4 knock-down and application of an anti-VLA-4 mAb reduced transmigration up to 40% compared with B16F10 cells.

Data are presented as mean values \pm SD ($n \geq 5$; *** $p < 0.001$ with respect to stimulated B16F10 cells).

Figure 2: P-selectin dependent interaction of B16F10 and B16F10 clone cells to platelets under static and dynamic conditions. **A)** B16F10, B16F10 control, or B16F10-VLA-4kd cells were incubated either with unstimulated or TRAP-6 activated platelets (1×10^8). Stimulated platelets showed an augmented adhesion to the cells. This interaction could be abrogated by adding a P-selectin binding mAb ($5 \mu\text{g}$) or Tinzaparin ($500 \mu\text{g}$), respectively. A control mAb ($5 \mu\text{g}$) had no influence on tumour cell platelet interaction. B16F10, B16F10 control, and B16F10-VLA-4kd cells showed nearly an identical adhesion behavior (**n.d.**, no differences between B16F10, B16F10 control cells, and B16F10-VLA-4kd detectable).

B) Detached B16F10, B16F10 control, or B16F10-VLA-4kd cells were incubated with TRAP-6 activated (a.p.) and resting platelets (u.p.) (1×10^8) for 5 min under gently shaking conditions and then determined by flow cytometry. B16F10 and both clones showed no differences in fluorescence intensity indicating similar platelet interaction capacities. Tinzaparin reduced the tumour cell platelet interaction approximating the behaviour of resting platelets. One representative of three comparable results is shown here.

Figure 3: Elucidation of VLA-4 mediated metastasis in an experimental mouse model.

Mice were killed 14 days after tumour cell inoculation into the tail vein (B16F10, B16F10 control, or B16F10-VLA-4kd cells) and pulmonary metastatic burden was determined. B16F10-VLA-4kd cells reveal a bisection of metastases compared to B16F10 cells.

Figure 4: Differentiation of VLA-4, P- and L-selectin contribution to hematogenous metastasis. B16F10 or B16F10-VLA-4kd cells were injected into P-sel $-/-$ mice and after termination the pulmonary metastatic foci were quantified. Some mice were treated with 67

IU of Tinzaparin prior to tumor cell injection. Mice administered with B16F10-VLA-4kd cells displayed a slight decrease in number of metastases due to VLA-4 knock-down and remaining L-selectin function.

Figure 5: Inhibition of metastasis due to Tinzaparin and N-acetylated heparin application. Wild type mice were intravenously injected with 67 IU of Tinzaparin or 150 µg of N-acetylated heparin, followed by injection of B16F10 or B16F10-VLA-4kd cells (1.5×10^5) through the tail vein 10 min later. After euthanization, for all three groups metastatic nodules were hardly detectable and tend to zero due to a P- and L-selectin, and VLA-4 inhibition by Tinzaparin and a P- and L-selectin blockade mediated by the N-acetylated heparin derivative.

Figure 1

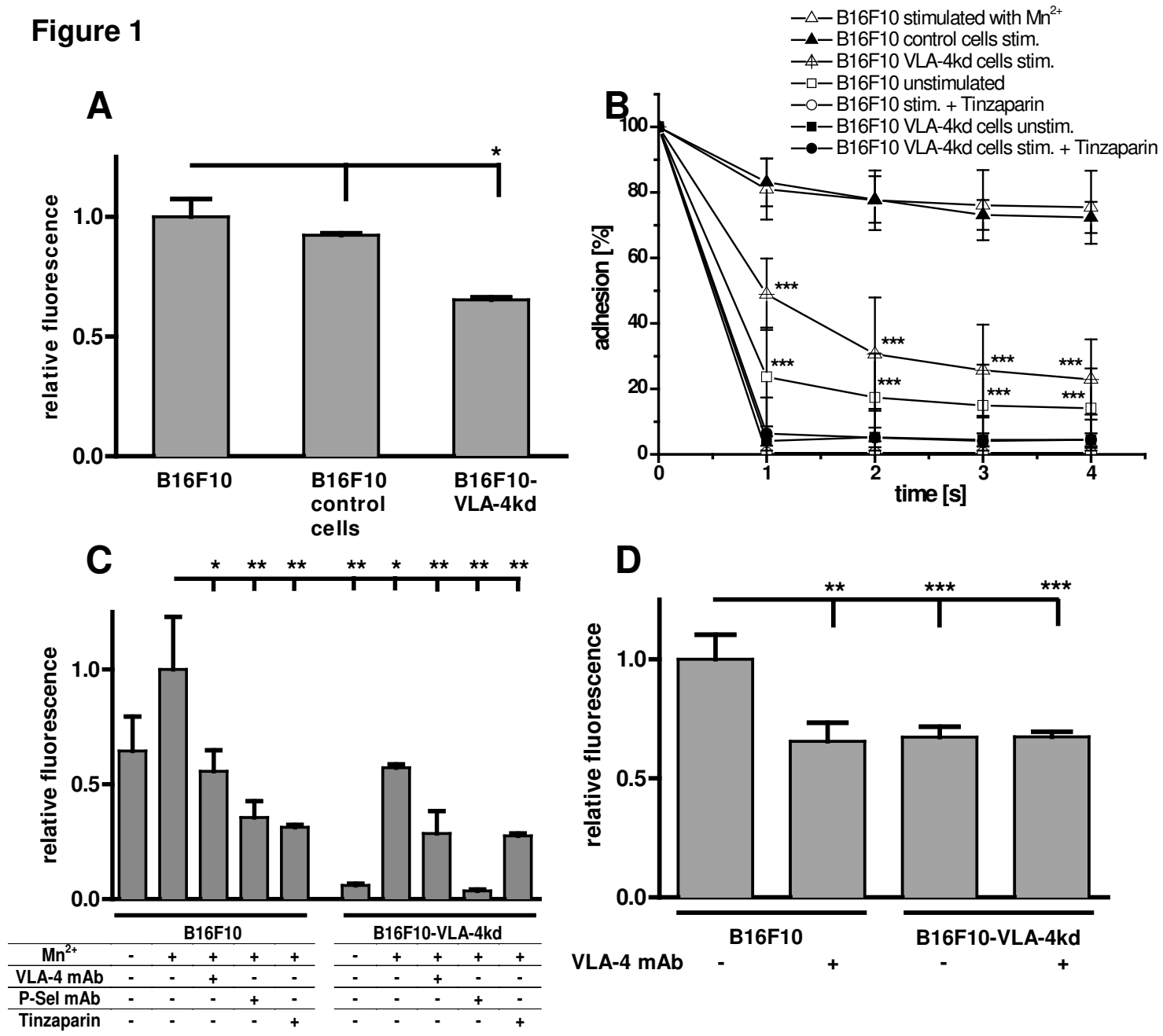
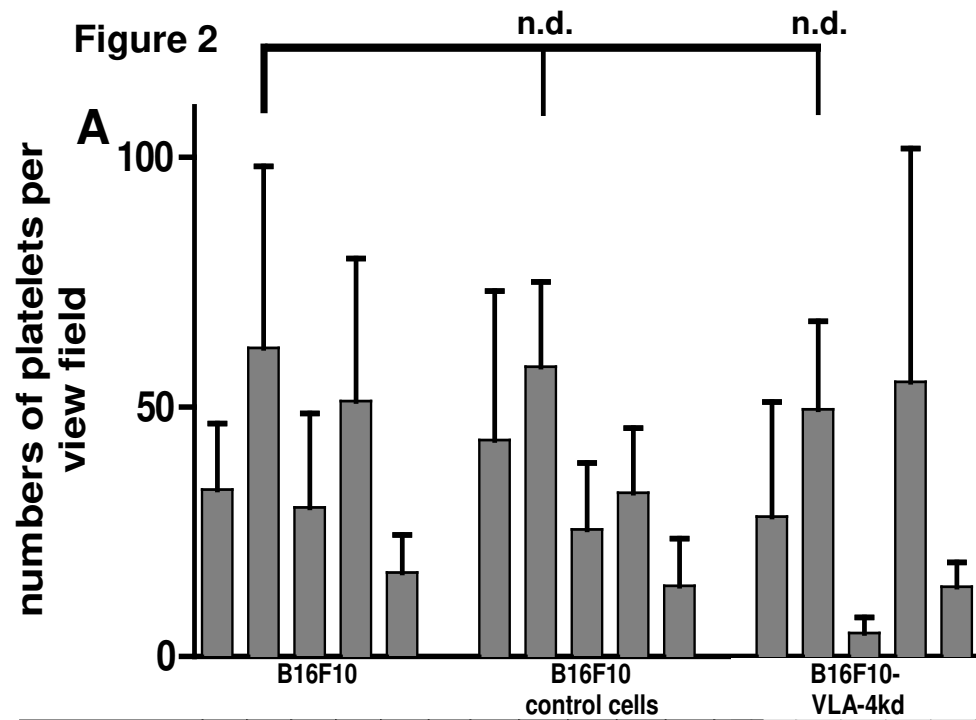


Figure 2



non-activated	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
activated	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
P-sel mAb	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-
Control mAb	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-
Tinzaparin	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+

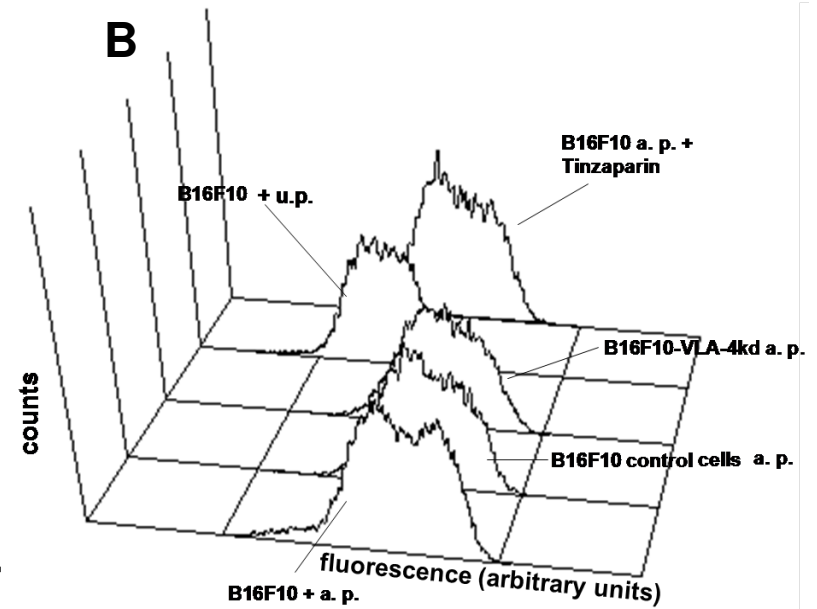
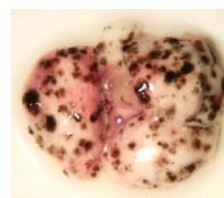
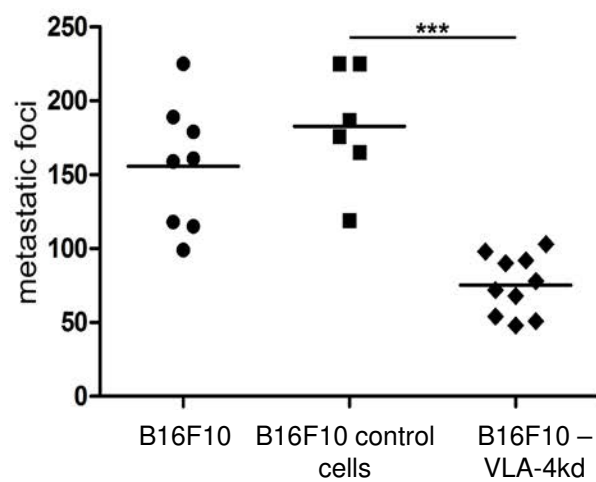
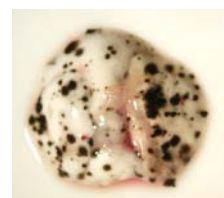


Figure 3



B16F10



B16F10 control cells



B16F10-VLA-4kd

Figure 4

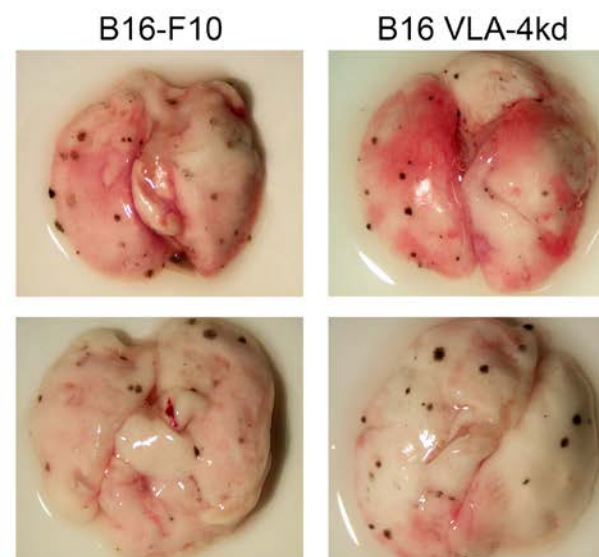
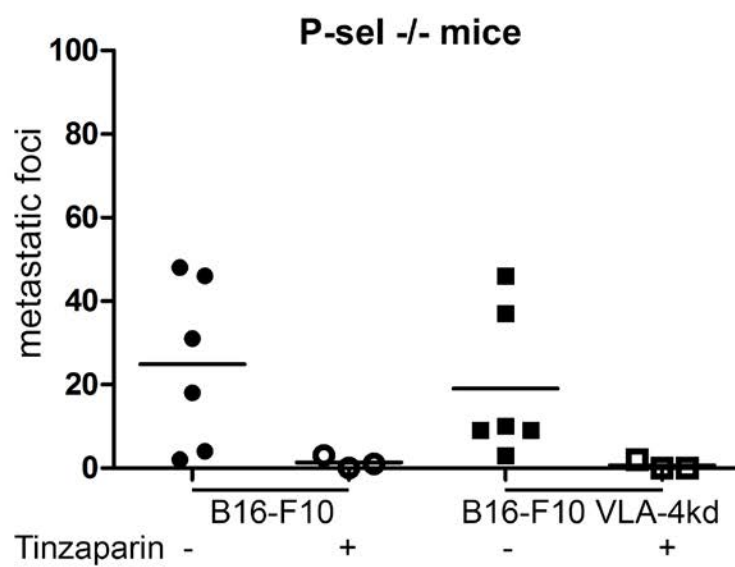


Figure 5

